EXPERIMENTAL METHODS USED IN DETERMINING CHRONIC TOXICITY

A Critical Review

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1. Outline of the Review

The evergrowing impact of the chemical industry on the technology of food production and processing has led to the accidental or deliberate addition of new

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chemicals to a variety of foods. There has been increasing concern lest the health of the consumers be in some, as yet unrecognised, way affected by these foreign chemicals. The possibility of chronic toxic effects arising from contact with chemicals has long been a problem of industrial hygiene. Those responsible for the health of either the general population or the groups of industrial employees exposed to chemicals have to decide what, if any, would be the largest quantity that could be absorbed with safety for a more or less indefinite period.

In industrial situations it may be possible to decide what is a safe or permissible degree of exposure on the basis of past experience and observations on the health of those who have been exposed. In the absence of such previous experience, those charged with the responsibility for the public health turn with increasing frequency to the experimental biologist for his opinion as to the likelihood of a certain chemical producing injury if absorbed in quantities not overtly toxic over long periods. It is obvious that absolute proof of the safety of a chemical for man will not be demonstrated by experiments on animals, but equally it is clear that some observations on animals must be made. It is the purpose of this review to examine critically the experimental methods that are used in this difficult type of work. Importance is often attached to the so-called "long-term toxicity test." Such tests for chronic toxicity are usually designed in order (a) to ascertain the dose that may be absorbed over long periods (½–2 years) without producing the signs of intoxication characteristic for the same compound when given in larger amounts, (b) to exclude the possibility that these subtoxic amounts will produce some hitherto unsuspected reaction in the experimental animal.

There is a growing tendency to rely upon this general type of "toxicity test" as evidence of the harmlessness of a new chemical. It may appear to allow a decision to be made that might better have been based on a fuller knowledge of the metabolism of the compound. This tendency to use "chronic toxicity tests" may lead to the suggestion that the type of test should be standardised. An initial review of some of the methods used or recommended for this type of work seems to be needed. No similar review has been found though there have been occasional doubts expressed about the wisdom of this type of experimental approach to a biological problem of extreme complexity. All the papers describing chronic toxicity tests have not been examined but a selection has been scrutinised with some care. Comment and criticism have been made in the hope that better experimental methods may eventually be evolved.

The proposals made by others for the form which such toxicity tests should take have been reviewed. Some are criticised on the general grounds of their practicability.

A more detailed examination of the experiments that have actually been carried out and reported has then been made. A number of factors that are, or should be, taken into account have been discussed and their relative importance in assessing the results of such experiments considered. The value or validity of some of the criteria of toxicity have been criticised. Consideration is given to the statistical significance of the results of the typical test of this kind.

The problem of interpreting the results of these experiments and applying the findings to the situation as it affects man is discussed. The conclusion reached is that the conventional type of toxicity test is an unsatisfactory approach to this problem. It is often an attempt to prove a negative, and very large numbers of animals should be used to make any such results statistically significant even for the species investigated.

This suggests that a different experimental approach be made to the study of chronic toxicity.

2. THEORETICAL CONSIDERATIONS

2.1. Desiderata for the Ideal Test for Chronic Toxicity

An acute toxicity test is normally designed to discover the dose needed to kill 50 per cent of animals or to produce an effect which is simple to measure quantitatively, or which is often recorded as simply positive or negative. For example, the effects of sex, species and age, the value of antidotes and prophylactic agents, the influence of diet, environmental temperature and route of administration may all be determined in a series of tests and the effective doses under each condition compared. This subject has recently been reviewed (118).

There are a number of long-term toxicity tests which are designed with similar specific objectives such as tests for carcinogenicity or tests for the effect of vitamin or mineral deficiencies and similar nutritional tests. The chronic effects of a compound may be assessed by making a specific measurement. The effect of feeding a compound suspected of having an action similar to that of thiourea may be measured by studying the weight of the thyroid. The effects of certain organo-phosphorus compounds can be related to changes in the activity of the cholinesterase in the blood and tissues of the animal ingesting them. This type of test is similar to the acute test except that the complication of the long time interval restricts the ease with which tests under different conditions can be carried out.

The experiments to be considered here rarely include any specific objective measurement of the effect of the material under test. The usual object is to find a "safe" or "harmless" dose that may be fed to animals over long periods. The measurement of non-specific effects is bound to be much less informative than that of specific reactions related to the biological behaviour of a compound. Although the evaluation of a response must be less precise, the object of these tests is often more comprehensive.

Almost anything will prove harmless if given in small enough quantities, but it is not always easy to define "small" quantities. Immediately an attempt is made to define a safe or harmless dose, it must be asked, "Under what conditions?" It should be safe to young and old animals including pregnant and nursing females, and sucklings. It should be safe under conditions of full abundant nutrition or where the diet is only just adequate. It should not enhance the susceptibility or reaction to common infections. It should not increase the incidence of other natural diseases and disabilities. It should not reduce the animal's

resistance to conditions of stress, *e.g.*, heat, cold, hard work, temporary starvation or deprivation of water. It is obvious that the task of defining a safe level of a chemical based on long-term toxicity tests designed to meet even a few of the conditions just mentioned would be time-consuming and difficult to carry out.

Some of the proposals that have been put forward for the design of such tests and the published results in such tests have been examined. Criticism is made in the hope that more work and thought may be put into the design of this type of test. The limited experience of the authors has made them aware of the difficulties of doing this kind of work.

2.2. Suggestions for the Design of Tests

Most of the schemes for chronic toxicity testing have been put forward in connection with the presence of chemicals in food. These tests will be dealt with in some detail. Inhalation experiments are considered separately (Section 3.3.4). The standard publication on the toxicity test is that of Lehman and his colleagues (183) which brings up to date their previous reports (50, 299, 309, 315). Other writers (110, 113, 115, 198, 297) for the main part reiterate the recommendations of Lehman *et al.* (183). Lehman *et al.* believe that the information required in order to assess the risk arising from the use of some material likely to appear in food can be classified under the following four headings.

1) Chemical and physical characterization of the material to be tested. This includes knowledge of chemical structure, purity, stability, presence of contaminants, etc., and of physical properties such as solubility and vapour pressure. An adequate analytical method for micro-amounts in biological materials is very desirable.

2) Acute toxicity. This is usually expressed as the LD_{50}, the amount of the substance expected to kill half of a group of animals. The material should be given in aqueous solution if possible, both orally and by intravenous injection (183, 113). Both the pure material and the finished commercial product should be given in order to study the influence of other ingredients on toxicity. At least three species should be used, one of them a non-rodent. These tests may throw some light on the mechanism and site of action of the material. This test may be of less value than the authors suggest because many of the compounds likely to be considered for use in food have no detectable toxic action and it is impossible to measure acute toxicity by this means.

3) Subacute toxicity. A test for subacute toxicity is designed as a pilot experiment to guide the planning of chronic tests, from which it differs only in magnitude and duration. Weanling rats are the animals of choice. Four groups of ten animals are used: (a) a control group, (b) a group on a diet containing 10 times as much of the ingredient as is proposed for use in food, (c) a group given the highest level the animals can tolerate, (d) a group given a dose between (b) and (c). Of course additional groups may be required. Smith (261) has used a simpler and more economical test. Dogs have been used as a second species and one dog is assigned to each of the four groups. The materials are administered for 2–4 months.

Observations are made on the rate of growth, food and water intake, fertility, mortality, general appearance and behaviour of animals, including activity and functional pattern (113). Blood and biochemical studies are made during the experimental period, and at the end of this time an autopsy is performed and the principal organs taken for histological section. The possibility of carcinogenesis should be examined (110, 297). Percutaneous tests on rabbits (110) a study of allergic responses (183) and other additional tests may be added.

The subacute test is in itself a formidable undertaking and indeed few substances receive any further study. The toxicologist in industry faced with the need to provide certain basic data rapidly and on a considerable number of compounds is forced to limit the scope of his investigations to subacute tests. Smyth and his colleagues have discussed the design of such tests and testified to their value (268, 270, 271, 275). It is in fact doubtful whether it is worth proceeding beyond the subacute tests.

4) Chronic toxicity. If the use of the compound has not been excluded on the basis of the above tests, a long term toxicity test is started (183, 297). It is recommended that two species of animals be used, the rat for its lifetime (set at 2 years), and either dogs or pigs for one year (297). Groups of rats containing at least 10 males and 10 females are distributed as follows: (a) controls (b) a group given a diet containing 100 times the concentration proposed for use in food (c) a group given a diet containing the highest tolerated amount (d) an intermediate group. The actual dose levels are of course based on information derived from the subacute tests. With the second species, 3 animals are put in each of 4 groups (a) controls (b) a group given a low level producing no damage (c) a group given a high level approaching the highest tolerated amount (d) a group given an intermediate level.

The criteria of toxicity that are examined are those listed in the subacute test, with the addition of nutritional studies, paired feeding, studies of metabolism of the material and so on. Some idea of the extent of these recommendations can be given by two quotations—"Depending upon the individual case these experiments may involve studies on isolated tissues or organs, careful pharmacodynamics, electrocardiograms, electroencephalograms, chemical antidotes, chemical studies of blood pigments, isolated loop or pouch studies, renal clearance etc." (183). Fraser (113) points out that if the material interferes with the nutritional value of the diet it is unlikely that this will be shown up with the ordinary toxicity test and more detailed studies are necessary. He adds "The route of absorption, final destination, storage, metabolism and excretion of the material should also be determined if possible. Radioactive isotope-labelled materials may be particularly helpful in studies on distribution and cumulative action."

A toxicologist obediently following these recommendations could scarcely say that his work lacked variety, or that he was not called upon to use to the full his skill and knowledge. In outlining the suggested schemes for chronic toxicity tests comment has been largely avoided. There is a fundamental confusion in these designs, that is well illustrated in the following quotation. "Complete study of the physiology, biochemistry and pharmacology of the substance under

test enables the chronic toxicity tests to be properly designed to cover any likely effect of the substance, directly or indirectly, on the bodily functions" (115). If such a complete study has already been made there would be little need for a chronic toxicity test. A chronic toxicity test is always a makeshift affair to be replaced as soon as possible by a more permanent structure of knowledge built on the foundations of physiology, biochemistry and the other fundamental sciences.

3. DESIGN AND EXECUTION OF CHRONIC TOXICITY TESTS

3.1. Introduction

In the following sections more detailed consideration will be given to the techniques either proposed for, or actually used in chronic tests. In each case the influence these factors may have upon the result is considered in relation to the difficulties their adoption may present for the proper execution of the tests.

3.2. Animals

3.2.1. Choice and numbers. The rat is the first choice because it is readily available, cheap, fertile, omnivorous and hardy, is well standardised and studied and has a short life span (16). Disadvantages such as its small size, the tendency for its food intake to be affected by small changes in composition, difficulties in obtaining adequate blood samples and a proneness to pulmonary disease do not weigh seriously against it. The only animals challenging its position are mice (44) (especially in carcinogenicity testing), and hamsters (61, 134, 197). Some workers have based their preference for the rat over the guinea pig and the rabbit for inhalation experiments on the much greater susceptibility of guinea pigs and rabbits to toxic pneumonia (28, 269) or the "functional similarity" of the rat to man (56). In most experiments the rat has been used because of its convenience and availability in the absence of any contraindications.

The number of rats in each group represents a compromise between a desire for a sufficiently large number of animals to allow statistical treatment of the data, and the need to reduce the amount of data to be collected so as not to overwhelm the investigator. In practice ten or less animals per group are used in about half the published tests and 30 or less in 90 per cent of the tests. If the sexes are to be treated separately the groups are reduced to 5 and 15 respectively. Some aspects of the problem of group size are discussed in Section 4.7.

Designers of chronic toxicity tests call for the use of a second species that should not be a rodent (see Section 2.2.). It has been argued that if the response in several species is similar the result can be transferred to man with greater confidence than where a considerable species difference has been detected. The choice of the second species is often based on a fancied similarity to man and various authors have recommended, the pig (81, 277) the dog, (228) and the monkey (269, 304).

The chief argument in favour of the monkey is that it is a primate. Monkeys are obtained by capture and are of uncertain age and temper. They require a period of adequate feeding, treatment for ecto- and endo-parasites, and even

courses of antibiotics before they can be used. Monkeys are difficult to handle and survive badly in captivity except in warm climates. They are particularly prone to tuberculosis and dysentery (73, 165, 289). Thus Robertson and his colleagues (236) lost 10 of 29 monkeys in experimental groups and 8 of 16 controls from disease in the first 7 months of a chronic toxicity experiment. The rhesus macaque (*Macaca mulatta*) has been used almost exclusively. Monkeys have a further disadvantage in that they are herbivores and very little is known of their nutritional needs (73). The administration of the drug may be difficult. Inhalation has been the commonest means of exposure (1, 2, 88, 193, 236, 240, 281, 289) but others have resorted to administration by stomach tube (282, 296) for periods up to one year. It is not surprising that only small numbers of monkeys have been used, *i.e.*, 1–6 in most experiments (1, 2, 19, 88, 169, 193, 289, 296) and these mainly over short periods. In a few experiments larger groups of 29 (236) 15 (265) and 10 (282) have been used, but apparently in no chronic toxicity test with monkeys have the recommendations put forward in Section 2.2 been carried out fully, and the same may be said for experiments with dogs.

Most laboratories use mongrel dogs of all sizes, ages and breeds. Factors such as differences between breeds, litters, sexes, etc. are completely submerged in the obvious heterogeneity of these animals and some of the simpler criteria of toxicity such as growth and fertility tests cannot be used with dogs. One solution to this difficulty would be to breed dogs especially for experimental purposes, but this is too expensive for most laboratories. Thus Robinson (94) stated in 1945 that it cost 150 dollars to raise a beagle pup to the age of one year. A toxicity test with mongrel dogs is a very different thing from a toxicity test with rats, and it is a common and rational practice to use dogs as individual experimental animals in special circumstances. These include experiments requiring repeated samples of blood or urine for chemical analysis during the course of chronic exposures (52, 145, 151, 169, 228, 232, 233, 255, 289). Experiments where a few dogs have been added just as another species have gained little by this addition. Where the number of species under test has been further multiplied there may be no advantage to offset the resultant complication. Thus an experiment in which 32 rats, 32 guinea pigs, 7 rabbits, 6 monkeys, 2 dogs and 8 cats were used (88) is exceedingly difficult to interpret. The same may be said for other experiments in which 4 or more species have been used (1, 193, 195, 221, 240, 278, 281). Most of these are inhalation experiments where once the apparatus has been set up no further modification is needed for each new species.

3.2.2. Species variations in susceptibility to toxic substances. The use of a second and non-rodent species in chronic toxicity testing has not received the trial desired by its supporters. The reasons for advocating the multiplication of species used in tests for chronic toxicity merit some discussion.

That different animal species may vary considerably in their susceptibility to the acute toxic action of chemicals would be generally accepted. Considerable differences are sometimes found as in the response to fluoroacetate (62, 63) or alpha-naphthylthiourea (211). Minor variations in the response of different species have been reported for such materials as chlorinated hydrocarbons

(240, 281), beryllium (285), insecticides (130, 214), acrylonitrile (88), atabrine (195), and indeed in almost every test where several species have been used. These findings are just another indication of biological variation that is no more surprising than the differences in physical appearance that enable the rat to be distinguished from the dog at a glance. However, there is not the same knowledge of toxicological response as there is of anatomical differences between the species. If the tests for acute and subacute toxicity are carried out on a number of different species, wide differences in species sensitivity to new compounds will be demonstrated. No experimental evidence available suggests that differences not revealed by this type of experiment will appear during the course of feeding tests extending over long periods.

It would be an advantage if the species chosen for any test for chronic toxicity could be shown to respond in a similar way to man. Unfortunately, there are no general rules which would enable the experimenter to make the proper choice in any particular instance. In comparing the response of man and animals to toxic substances it is unusual, except in the case of drugs given therapeutically, for the dose taken by man to be known. A fair estimate may occasionally be made as in an accident resulting from the use of sodium fluoride instead of dried milk powder in making a food for hospital patients (188). In the disaster following the use of diethylene glycol in a sulphanilamide preparation it was very difficult to estimate the dose taken, or to correlate it with the effects suffered (51). A comparison has been made between the susceptibility of rat and man to a number of metallic poisons by comparing the toxic doses expressed on a body weight basis. The following figures were derived (93):

ELEMENT	POISONING	FRACTION OF TOXIC DOSE FOR RAT PROVING POISONOUS TO MAN
Arsenic	Acute	1/5
Barium	Acute	1/10
Copper	Acute	1/5
Lead	Acute	1/25
	Chronic	1/150
Mercury	Acute	1/3
Radium	Chronic	1/150
Selenium	Chronic	1/60
Thorium	Acute	2/5

The biggest differences relate to chronic poisoning. While this may reflect the difference in criteria used to assess toxicity in the case of lead and selenium, in the case of radium the criterion was the same, namely, the development of bone tumours.

In its response to the demyelinating effects of triorthocresyl phosphate (262) and other organophosphorus compounds (27) the chicken more closely resembles man than the other species tested. There is no known biological reason for this. No one has advocated the use of birds in tests for chronic toxicity.

Unfortunately, some of the most serious of the chronic effects of toxic sub-

stances are apparently seen only in man. Thus many compounds of diverse chemical structure will produce, in a proportion of those taking them, aplasia of the bone marrow. Benzol will produce such a condition whenever it is absorbed in large amounts by both man and experimental animals. Sporadic cases of agranulocytosis have not been encountered in animals given drugs that will produce the condition in man.

Dinitroorthocresol, like dinitrophenol, produces an acute toxic effect that is similar in a very wide range of living matter (222). The acute toxic dose for man is close to that for laboratory animals (123). Repeatedly administered it will produce permanent and serious lens opacities in about 0.1 per cent of those taking the drug (157). It was discovered quite accidentally that when fed to ducks and ducklings these drugs will also produce lens opacities (234). These lesions are acute. If the drug is injected opacities appear in a few hours and disappear equally rapidly (89a).

The incidence of agranulocytosis and lens opacities is probably of the order of 1 in 1000 of those taking the drug. There has never yet been a chronic test on animals carried out in sufficient numbers to exclude the possibility that they are equally susceptible to such an occasional complication. Reference to Section 4.7. will show that it would be necessary to use groups of 300 animals in order to be able to detect an incidence of 1 per cent.

A second species is never likely to be used in numbers approaching those needed to detect reactions with a low incidence rate.

A more rational use of different animals should be made, such as the use of dogs when repeated samples of blood or large amounts of tissue are required for analysis, of monkeys for inhalation experiments because of anatomical similarities to man, of chickens for a study of demyelinating agents, and of cats for compounds likely to produce methaemoglobinaemia (186). The investigator is rarely so completely in the dark about the possible mode of action of his compound that he cannot be guided in his choice of animals by his own experience and by the reports of others.

3.2.3. Genetic features. If it is agreed that for many experiments the rat is the animal of choice, some consideration is needed of the strain of rat to be used. There are a number of strains and species of rats of which the most widely used are the albino strains of *Rattus norvegicus*. This choice raises its own difficulties. Darwin originally pointed out that the domestication of animals has led to a great variety of strains whereas the wild population is remarkably uniform. It is likely that the variation in response to drugs from one rat colony to another may be much greater than the natural variation in the wild rat. This conclusion is supported by observations on the acute toxicity of thiourea (80). It was found that one stock laboratory strain of rats was 160 times as sensitive as a second strain. On the other hand, wild rats whether of Alexandrine or Norwegian species were very similar in their response to thiourea and were 300–400 times as resistant as the most sensitive laboratory strain.

Despite this warning of the difficulties produced by selective breeding, there is a tendency to strive for greater uniformity of response in rats used in chronic

toxicity tests. This is probably a heritage from biological assays where attempts are made to exclude variability other than that produced by the drug being assayed. Thus Bacharach and Chance (21) found significant inter-litter differences in the assay of gonadotrophin and suggested the advisability of a symmetrical distribution of litter mates in the various groups in other biological assays. However, even in biological assays the value of this practice is not proved for Gaddum (118) has stated that there is little or no direct evidence that genetic homogeneity itself increases the uniformity of response to drugs, and that it may even be harmful. Emmens (92) was surprised to find that a highly inbred albino strain of mice was more variable in response to oestrone than randomly mated albino mice. He concluded that the factor causing variation in response to oestrone was unlikely to be dependent on differences in genetic constitution.

The common belief that genetic variability is eliminated by using litter mates as experimental and control animals is true only of litter mates of a highly inbred line and does not hold good for others (159). Litter mates may differ as much as any two siblings born months or even years apart. As it takes about 16 controlled matings to obtain almost complete homozygosity and this may be upset at any time by mutations, few laboratories are able to maintain a sufficiently inbred stock to make the extensive use of litter mates worthwhile. The use of inbred strains may be called for in such tests as for carcinogenicity (71, 260) but it is probable that uniformity of response is often sought as an end in itself. Thus, Lorenz and his colleagues (190) in studying the biological effects of gamma radiation, found with inbred guinea pigs very steep dose-mortality curves, but with hybrid guinea pigs the curves were less steep and there were some very resistant animals. This was taken to indicate "the importance of the use of inbred animals for obtaining reliable quantitative data." It is difficult to see why the data from the hybrid guinea pigs were less reliable, and the casual reader may well be more interested in the resistant animals than he is in the shape of a curve.

However, some workers have found significant inter-litter differences in chronic toxicity tests (100, 103) and others have also been careful to distribute litter mates through the groups by acceptable statistical methods (7, 199, 205, 241). This is a reasonable practice where the experimenter breeds his own animals on a sufficient scale and can have confidence in the litter mates. But when the significance of the results has to be assessed in relation to other species, and particularly to man, the unknowns are so great that details such as inter-litter differences are swamped.

3.2.4. Choice of sex. Designs for chronic toxicity tests call for separate treatment of the two sexes and in many toxicity tests this duplication is accepted without question. This is probably another legacy from biological assays. A list of sex differences has been compiled by Holck (153) but an example of the magnitude of these can be obtained from the statement by Bliss and Cattell (38) that red squill in rats affords a striking example of a sex difference in susceptibility since it is approximately twice as toxic to females as to males (191). This sort of difference may well be remarkable in biological assays where the LD_{50} or ED_{50}

may be expressed to three significant figures. Such accuracy is beyond the scope of the chronic toxicity test where only a few dose levels can be used and these widely spaced often as a logarithmic series such as 1, 10 and 100 parts per million.

Where sex differences have been recorded they usually consist of a difference in response at a single dose level. It is profitable to examine to protocols of a few representative examples that have been quoted by Holck (153).

1) Sex difference in selenium poisoning (100). Wheat containing 3 ppm of selenium was fed to rats. At six months, the mortality in males was 11 per cent, and in females was 60 per cent. However, in another experiment recorded in the same paper females fed on corn containing 5 ppm had a mortality of only 17 per cent after 12 months.

2) Sex difference in cadmium poisoning (162). At a dose level of 125 ppm of cadmium three female rats lived about four times as long as six male rats. However, two male rats on 500 ppm survived almost as long as 2 females on 250 ppm and certainly much longer than the 6 male rats on 125 ppm.

3) Sex difference in resistance to sulphanilamide (170). A single dose of 2.5 gms. of sulphanilamide per kilogram given subcutaneously killed 59.1 per cent of male rats and 95.7 per cent of females. Similar treatment of rats with an acquired tolerance to sulphonamides killed 37.5 per cent of the males and 81.8 per cent of the females.

The difficulties in interpreting sex differences in response can be illustrated by reference to chemical carcinogenesis. The recognition of the carcinogenic effect of certain hormones has led to detailed studies of the influence of sex on the development of tumours. Some workers have found no significant sex differences (39, 42), whereas others (18) find sex differences vary with the type of carcinogen and depend for their detection on careful control of the dosage.

Some well-substantiated cases of sex variation in response are found in rats treated with the short-acting barbiturates where the effects of spaying and the administration of sex hormones have been studied (55, 149, 154, 155, 156, 286). Male and female rats also differ in their susceptibility to parathion (p-nitrophenyldiethylthiophosphate) and schradan (octamethylpyrophosphoramide). The female is more susceptible to the former, but less susceptible to the latter (5). This difference is not a reflection of the susceptibility of the sexes to inhibitors of cholinesterase for no such difference is seen in their response to compounds such as tetraethyl pyrophosphate. The differences in the response to parathion and schradan are probably related to the facility with which the sexes produce or eliminate the inhibitor formed from the drug administered (5).

There seems to be little ground for conducting chronic toxicity tests on equal numbers of each sex as a routine. From the practical point of view limiting the experiment to one sex halves the number of animals available from the stock colony, but since the sexes must always be treated separately in studying factors such as growth, this does not mean that fewer animals are available of whatever sex is chosen. Unless reproductive studies are to be included, it may be better to use larger groups of one sex in a given experiment.

3.3. Administration of Compounds

3.3.1. Purity. An examination of the toxic properties of a chemical entity would presumably be done with a pure specimen of the material. While absolute purity would be rare, one should be able to assume that those impurities that were present were not in concentrations capable of exciting any biological response.

It is however rare for chemicals used on a commercial scale to be pure, and the problems raised by the presence of toxic impurities have long been familiar to the industrial toxicologist. The nature and quantity of any impurities will depend upon the method of production and the composition of the raw materials that are used. For this reason the hazards in making a given material may vary from one place of manufacture to another. This situation presents a dilemma to the experimental toxicologist trying to assess an industrial hazard. If he uses only the one material manufactured by the enquirer his findings may be relevant only to one particular situation. On the other hand the use of a carefully purified material may give an equally unrealistic picture of the toxicity of the material to which the workers are exposed. However, any experimental data obtained with pure material will at least have some scientific value. There is no simple solution to this dilemma, but a decision whether or not to take steps to secure pure material for a test might rest on the following considerations. If the experiments planned are of the general type in which only crude criteria of toxicity are to be employed it matters little whether pure material is used. The answer to the *ad hoc* problem will best be supplied by the use of the actual material to which human exposure occurs. If any precise or time-consuming measurements are to be made it seems desirable to use purified materials so that the results of any careful and perhaps onerous laboratory investigations may be applied by others.

Ideally perhaps the toxicity of each ingredient of a mixture should be assessed together with that of the mixtures that occur in manufacture. This sort of analysis could be done by means of acute toxicity tests, but the tests for chronic toxicity are so imprecise that any attempt to carry out such detailed analysis would probably be a waste of effort.

3.3.2. Doses. Whenever an experiment is planned to run for at least a year, the decision about the doses to be administered assumes considerable importance. Only at the end of the experiment may it become possible to decide whether or not a good decision was made. The plan of the experiment would be upset if, during its course, the dose was altered.

The following are typical examples of what have been suggested as satisfactory dose levels:

1) Doses should vary from those showing no effect to those producing marked lesions (315).

2) Three or four levels differing by a factor of 2 or 4 should be used with the least dose adjusted so as to produce no effect (271).

3) Three dose levels should be used. In terms of quantity administered per unit body weight, the lowest dose should be the equivalent of 10 times the calculated daily intake of man. The greatest dose should be one that produces some

toxic reaction and the third dose should be an intermediate one determined by the experimenter (183).

4) The animals should be given doses, calculated on a body weight basis, up to 100 times greater than the estimated daily intake by man (113, 114).

Recommendations 1) and 2) cover any toxic material administered orally or by inhalation, but 3) and 4) refer to tests on chemicals that may be used in food.

An analysis has been made of the reports of 33 tests carried out in different laboratories over a number of years. A single dose level was given in 3 experiments. No reason for the selected level was given in one (87); in another it was arbitrarily selected as 30–40 times the maximum permitted concentration (228) and in the third it was said to be 1000 times the likely daily intake of man (242). In 13 experiments three dose levels were used and in the remainder 4–10 different doses were given. In the four experiments in this group done in the laboratory which made recommendation 3) above, 4 or more dose levels excluding the controls, were always used. The dose levels increased by geometrical progression in 18 experiments, by arithmetical progression in 3, and in irregular steps in 10.

Almost invariably tests for chronic toxicity follow those for acute and subacute toxicity. The dose levels used in these long-term tests have probably been based on the observations made during the preliminary shorter tests coupled with the knowledge and experience of the experimenter. The number of dose levels used in a test for chronic toxicity may perhaps be in inverse proportion to the previous work and experience of the experimenter.

Those who seek a scheme for arriving at the appropriate dose levels for this type of experiment should consult the paper by Smith (261).

In feeding experiments the dose levels are commonly given as parts per million of the diet or percentages when higher concentrations are used. For the rat a level of 1 per cent in the diet constitutes a dose of about 0.6 g/kilo/day, and 1 per cent in the drinking water about 0.8 g/kilo/day (271). Calculations such as these assume that the palatability of the diet or water has not been altered. The dose will also be determined by the age and sex of the rats (see Section 3.3.3).

In inhalation studies the dose is expressed as the concentration in the inspired air. It takes no account of the amount actually absorbed nor, in the case of particles, can it be assumed that the route of administration is confined to the respiratory tract. These points are discussed in Section 3.3.4. The duration of exposure is commonly fixed at 6–8 hours daily for 5 days a week.

There is no simple solution to the problem of deciding on dose levels for long-term experiments. A large dose range means many small groups or unmanageable numbers of animals. A small range calls for skill and luck in reaching a correct decision with the chance of much wasted time and effort. These factors provide one of the most serious arguments against experiments of very long duration.

3.3.3. Oral route. The route of administration has a considerable effect on the acute toxicity of drugs (38, 153) and its effect is no less important in chronic toxicity tests. The difficulties will be discussed in relation to the commoner methods of administration.

TABLE 1

Relation of Food Intake, Nominal Dosage and Actual Dose Consumed, in an Experiment in which Cadmium Salts were Added to the Diet (162) of Rats

CONC. IN FOOD (parts per million)	WGT. OF FOOD CONSUMED PER RAT PER DAY	WGT. OF CADMIUM INGESTED PER RAT PER DAY
	gm	mgm
1000	3.0	3.0
500	4.5	2.2
250	4.0	1.0
115	6.4	0.8
Controls	9.5	0

1) Addition of the test material to the food. This is the most widely used method as well as the simplest and most convenient, reducing the need to handle animals unduly and saving the time of skilled personnel. The material is mixed with the food to give the desired concentration and the animals are allowed to eat as much of the mixture as they wish. If the test material is insoluble in water, there is a choice of mixing the dry powder with the food or of dissolving the material in a solvent such as an edible oil and then mixing the solution with the food. The use of the solvent may alter the toxicity of the material (140, 153). Other difficulties arise as a result of mixing the test material with the food.

(a) The diet may be made unpalatable. Rats are suspicious of any change in the composition of their food, so that food intake may become erratic and less than optimal, especially at the start of an experiment. Other animals such as rabbits and cats refuse to eat unpalatable food. When this happens some alternative method such as administration by stomach tube must be used. Dogs frequently vomit up any material that upsets them (229).

Even if gross disturbance is avoided, relatively minor changes in food intake may affect the actual amounts of drug consumed. In Table 1 are listed the daily food intakes of rats given different levels of cadmium salts in their diet (162). At all levels there was some restriction of food intake, so that the dose of cadmium received by each group was not proportional to the concentration in the diet.

(b) The intake of food is not uniform over the period of the experiment. Rats eat much more relative to their body weight in the first few weeks after weaning than at any other period (241, 316). In grams per kilo body weight the daily intake falls from 150 at 6 weeks to 75 at 18 weeks. If the test material is mixed with the food, so as to maintain a constant concentration, the daily intake of the drug in the first few weeks may be at least double the intake between 9 and 12 months. It is during the first three months of a toxicity test that most of the casualties occur and these can be explained, at least in part, by the relatively high intake of test material at this time.

The sexes also differ in the amount of food that they consume and hence in the amount of test material that they ingest. Fitzhugh and Nelson (98) found that female rats were slightly more susceptible than males to DDT (dichlorodi-

phenyltrichloroethane) fed in this way and they attributed this to the 20 per cent higher intake of food containing DDT by the females.

(c) Adding the test material at high concentration to the diet may have an effect. In Section 2.2 mention was made of the recommendation for giving animals a diet containing 100 times the concentration of a chemical likely to be found in human food. Attempts have been made to test the toxicity of the material by adding as much as 25 per cent to the food. These materials include cellulose derivatives (29, 74, 150), surface active agents (135, 136, 137, 254) and anti-oxidants (78). Rats often need to be specially trained to eat these grossly altered diets (136). In most cases where the test material reaches a level of 10 per cent or more in the diet, growth has been affected and this has been due, at least in part, to the satisfaction of the animal's appetite by a large bulk of inert material. In the face of a positive effect on growth most workers have attempted to show that this is due to a decreased intake of actual digestible material rather than to a direct toxic effect on the tissues of the animal. This has usually been done by paired feeding experiments (29, 135, 257), or by calculating the efficiency of food utilisation from measurements of growth and food intake (109, 136, 254).

(d) The test material may remove essential nutrients from the diet. Rancid fats may inactivate vitamins and other essential growth factors; sulphonamides and other antibiotics may interfere with the intestinal flora; solvents such as liquid paraffin may reduce the absorption of the fat soluble vitamins (8); minerals may be rendered insoluble and excreted, as for example when feeding beryllium oxide produces rickets by decreasing phosphate absorption (6, 43).

The foregoing will indicate that while the method of adding the test material to the food has the advantage of simplicity, this is offset by a number of disadvantages. One additional measurement, that of food intake, is necessary, and exacting experiments such as paired feeding may have to be done in order to clarify problems arising from this method of administration.

2) Addition of the test material to the drinking water. This is suitable for water-soluble materials and has been used for dyestuffs (126a), germicides (219), glycerine (15), detergents (303), the ethylene glycols (131, 272, 273) and other alcohols (40, 184). With this method the intake of the test material is also poorly regulated and it is necessary to measure fluid intake. Boughton (40) found that rats restricted their fluid intake to 60 per cent of normal when alcohols were added to the drinking water in concentrations of 5 per cent. The rats receiving the alcohols were obviously thirsty because they gathered about the drinking bottles after each filling and left without drinking. Weight reductions of 20–30 per cent were evident in the alcohol groups at 9 months. On withdrawing the alcohols the fluid consumption was at once increased by 37 per cent in the ethyl alcohol group and by 48 per cent in the isopropyl alcohol group. These animals soon reached the same weight as the control rats, and Boughton concluded that dehydration played a very important part in the initial lag in growth in the experimental groups.

3.3.4. Inhalation. The inhalation route has been used in experimental studies

on chronic toxicity where this has been the way in which industrial workers have been exposed to a possible hazard. The effect of the material may be either a local one on the lungs, a general systemic one or a combination of both. Animal experiments have sometimes been used as a basis for setting up permissible limits for atmospheric contamination by vapours or toxic dusts. Animal experiments may be used to compare the toxicity of different solvents, and when these are volatile solvents experiments by inhalation are often used.

This brief review of some of the technical problems connected with this type of experiment is designed to draw attention to their existence and refer the reader to better sources of information.

The structure and function of the lower respiratory tract is remarkably uniform in the usual laboratory animals and man. The rate of air exchange is related to the basal metabolism which is in turn related to surface area rather than body weight. If the dosage administered to species of very different sizes is expressed as the concentration in the inspired air, it cannot be compared on a simple body weight ratio as is so frequently done when the oral route is used. Air intakes in the common laboratory animals have been conveniently collected in a nomogram (117).

In its behaviour as a filter the upper respiratory tract of the usual laboratory animals is very similar. It has been shown experimentally that the maximum size of particle that will pass through the upper respiratory tract of rabbits, cats, mice, rats and monkeys is similar (317a). The size is of the same order as that observed in man (174). Apart from the care needed in determining the dosage received, the use of the inhalation route for particles or vapours does not add to the already existing difficulties of extrapolating results from species to species including man.

Technically this type of chronic toxicity test requires more care than tests using the oral route but all the other problems such as those discussed in Sections 3.1, 3.2, 3.3 and 3.4 remain the same.

If it is the object of the experiment to study the effect of the particulate clouds on the lower as well as the upper respiratory tract and to include effects from absorption through the lungs, care must be taken to measure the concentration of respirable particles. The behaviour of particles of different sizes has been reviewed recently (175, 312) and it should be remembered that in discussing particle size in relation to this problem it is assumed that particles of unit density are being considered.

The various methods that have been used for producing dust clouds of suitable particle size have been briefly and clearly reviewed in a paper which describes an improved method for producing clouds of suitable selected particle size (317). The techniques for setting up mists and aerosols have also been discussed (239).

If the particles are too small—less than 0.5 micron—the greater part are exhaled again, but if they are too large—5 microns or over—they never penetrate the nose but are held up there and later swallowed. Even when care was taken to use only particles of respirable size, the prolonged inhalation of a mineral oil mist by monkeys produced pathological changes in the mucosa of the stomach in

addition to the injury of the lungs. The gastric lesions presumably resulted from the oil that had been caught up in the nose and swallowed though in these experiments it may also have been licked from the animals' coats (193).

Men exposed to dusts and particulate clouds will also swallow at least some of the material held up in their nose and in this respect the experiments simulate the natural conditions. However animals rely upon different methods for keeping themselves clean, and if steps are not taken to prevent contamination of their coats during exposure, large quantities may be ingested from licking their fur. Ingestion from contamination of the clothes of the industrial worker is unlikely to be more than that resulting from casual contact with food or cigarettes.

One method has been described whereby dogs could be exposed for 6 hours a day on successive days by introducing their muzzles only into the air stream containing the toxic agent (227).

If it can be assumed that the experimental conditions will ensure that the principal route of administration is by inhalation then it will also be necessary to provide proper methods of sampling the particulate clouds and determining particle size. Only if this is done can valid comparisons be made between the behavior of different materials. No conclusions can be drawn from experiments where such data are not provided.

The exposure of animals to vapours presents fewer technical problems, and there are a great many methods used for producing constant known concentrations of vapour. The exact technique used in any case will depend upon the physical properties of the material used. A few typical examples can be cited (161, 194).

It is usual to use the physical constants, *e.g.*, vapour pressure to derive a theoretical value for the concentration of the vapour added to the air flowing into a chamber in which the animals are exposed, but it is always essential to carry out analyses of the actual air in the chamber. There are a number of methods of continuous analysis. All modern methods of exposure depend upon the exposure of the animals to an air stream. The older method of volatilising a known weight of material in a large static chamber of known capacity cannot be used for exposures of anything but a very short duration.

The usual procedure is to expose animals for 6–8 hours a day 5 or 6 days a week for periods extending up to a year or more. In a few cases the animals live in the cages in which they are exposed except for short periods each day when the cages are cleaned. It is usual for control animals to be handled in the same way by being put in chambers through which air is flowing. In some cases a second control group may consist of animals kept in the animal house the whole time (2).

The criteria of toxicity used have been the same as those used in feeding experiments. Only exceptionally have the experiments run for more than 6–9 months. The incidence of pulmonary disease in rats of advanced age would make them particularly unsuitable for very long-term tests of this kind. It is usual for a number of species—rats, mice, guinea pigs, rabbits and monkeys—to be used but rarely have the groups been large. An exception was the study of toxicity of

a series of chlorinated naphthalenes where groups of 80 rats were used in many experiments (87).

In a recent series of papers (2, 3, 243, 281) the effect of a number of solvents has been studied on animals exposed daily for periods of 6–9 months. In some cases there appear to have been serious outbreaks of pulmonary infection in the rats and the conditions of the experiment would certainly favour the spread of an epidemic of this kind. It has not been possible to find a record of experiments of this type where an entirely unsuspected injurious effect upon the lungs was discovered. In the great majority of cases the inhalation of the vapour of toxic organic solvents seems to have been without effect upon the lungs even where extensive liver lesions were produced (31).

The attempt to produce pulmonary neoplasms by the inhalation of suspected carcinogens has been so unsuccessful (266) that it seems unlikely that by such experimental methods will unsuspected carcinogenic properties of a material be discovered. Inhalation may sometimes be a convenient way of administering a very volatile substance over long periods to animals. On the whole, however, there seems to be little point in using such a technique for making comparative determinations or in the search for systemic effects when the technically simpler feeding test would provide just as valid data on toxicity (87). The experimental production of pneumoconiosis is a special subject that is not considered here but reference may be made to a recent review (167).

3.3.5. Other routes. Toxicity studies by means of skin application are of short duration, but some, such as tests on insect repellents (82) and insecticides (83) have lasted as long as three months (see Section 5.4).

Many of the other methods of administration involve repeated manipulations and much handling of animals. Intravenous injections have been used for plasma substitutes (216); intraperitoneal injections have been used for antibiotics (238) and hypnotics (149). Usually these special methods have been used only over periods of a month or so but Thienes *et al.* (295) injected rats subcutaneously twice daily for a year.

Where difficulties have been met in feeding the material it has been given in capsules (171, 213) or more frequently by stomach tube to rats (4, 278), monkeys (265), rabbits (201, 272) and dogs (195, 201). This modification of technique may influence the result that is obtained. Nelson and Lyster (219) found that a higher toxicity was observed when a compound was given by stomach tube than when it was mixed with the food or the drinking water, probably because the full daily dose was given at one time by stomach tube. Repeated handling may upset the animals and there is always the chance of accidently passing the material into the lungs (160, 224, 247). Salomon and Cowgill (247, 248), faced with the problem of determining the toxicity of solder, developed a method of passing small pellets down stomach tubes into rats, and checked the number, location and excretion of pellets by x-ray examination.

3.4. Feeding

3.4.1. Nature of diet. The nature of the diet profoundly affects the results of both acute and chronic tests (85, 153, 258, 313). Thus a high fat diet increased

the toxicity of 2,4,6-trinitrotoluene (148) but decreased the toxicity of cyanide (200), fat augmented the acute toxicity but diminished the chronic toxicity of rotenone (12). The addition of DL-methionine or a combination of L-cystine and choline lowered the susceptibility of rats to the toxic effects of inhalation of 1,2-dichloropropane (146). The calcium and phosphorus content of the diet profoundly influenced the toxicity of lead and arsenic (49, 128). In almost every experiment where the effect of diet on toxicity has been studied findings of this sort have been recorded. Negative findings, for example, the report that dietary changes had no effect on the sensitivity of mice to senna (141), stand out as curiosities.

In some cases there is some indication of the way in which diet influences toxicity. When quinacrine was added to the diet at 100 parts per million, rats on low protein diet showed a retardation of growth while rats on a high protein diet grew normally. Measurement of the food intake showed that the low protein rats ate much more than the high protein rats and hence received a considerably higher dose of quinacrine (103). In other cases the addition of extra fat may improve the absorption of materials such as DDT (250), phosphorus and rotenone (14). Paraffin oil may take up certain vitamins in the intestinal tract and lead to their excretion in the faeces (153). Sulphonamides added to the diet disturb the microbial flora of the rat intestine and appear to inhibit the synthesis of growth factors (35, 133, 306). Harris and Kohn (133) showed that when rats were fed sulphonamides, the addition of the faeces of normal rats to the diet increased food consumption and restored the growth rate to normal. Coprophagy is apparently a factor in the nutrition of rats and it has been shown that when this has been prevented, growth on a full diet is less than in rats free to eat faeces (124). However the serious confinement imposed on rats in these experiments may play a part in decreasing appetite and growth.

The influence of dietary changes on chemical carcinogenesis is equally complex. Dietary effects have been found particularly with azo dyes (22, 65, 68, 204, 314) and to a lesser degree with the polycyclic hydrocarbons (41, 244, 287). Despite extensive studies no simple relationship has yet been found between diet and the carcinogenic potency of the azo dyes (168). The importance of the problem is shown by the evidence that primary cancer of the liver in man is determined, at least in part, by the nature of the diet (245).

In the ordinary chronic toxicity test the problem has been simplified by the extensive use of commercial diets which are based on modern knowledge of animal nutrition. The diets are aimed at producing optimum growth. This has been defined as the physiological state in which the animal functions in accordance with its design; actual achievements fulfill all the potentialities of the organism, and the growth curve is a pure expression of the inherent growth characteristics (89). This should provide a uniform background for the study of toxic effects but as a somewhat Utopian, or even Stakhanovite, concept it can be attacked at several points. A change from one presumably complete diet to another caused a 15-fold increase in the resistance of one strain of rats to thiourea (80). Wilson and De Eds (313) studied the effects of three different laboratory diets, all thought to be reasonably adequate, and found that the toxic dose of four different materials

varied considerably and unpredictably. They suggest that the use of a sub-optimal diet might provide a more sensitive index of toxicity, or that, as a compromise, the diet should be adequate but close to the borderline. The importance of the constitution of the diet may depend on the action of the toxic substance being tested. Given by mouth, the soluble salts of beryllium are not toxic because the metal combines with free phosphate in the diet and is precipitated. A stock diet might have a great excess of free phosphate or it might have an optimal quantity for full growth. The addition of beryllium would decrease the value of the latter diet, but not of that which contained excessive phosphate.

It is by no means certain that optimal growth is synonymous with optimal health. McCay (209) has observed the beneficial effects of restricted caloric intake on the survival of rats and their resistance to disease. He points out that with a food intake 10 per cent below that of a diet offered *ad lib.* rats will survive up to 1000 days (208), *i.e.*, about a year longer than the average expectation for rats. Low caloric diets delay terminal diseases (210) and degenerative conditions in animals (251). Schneider (252) has reviewed the growing literature supporting the general thesis that nutritional deficiency can lessen the susceptibility of the host, to infections by protozoa, bacteria and viruses. Further, Tannenbaum (292) has found that by restricting the caloric value of diets fed to mice to 80 per cent of the maximum he could lower the incidence and prolong the latent period for both induced and spontaneous tumours. From a study of the statistics of cancer mortality for man, he found that overweight persons had an increased susceptibility to cancer (291). The life insurance actuaries have long recognised that a weight below average in the second half of the life span increases the expectation of life.

3.4.2. Food intake. In Section 3.3.3 the effect of adding the test material to the diet was considered. Reduced food intake is one of the commonest factors depressing the growth rate, and measurements of food intake are commonly used to supplement growth data. In some cases both growth and food intake are unaltered (10) although when 5 per cent of inert material was added to the diet the animals had to eat a proportionately greater bulk to obtain the same amount of food (274). In other experiments where growth has been affected this has been associated with a reduction in food intake (9, 13, 46, 199). Depression of growth without significant change in food intake has been caused by synthetic sweetening agents (105), ethylene glycol (131), sulphonamides (133), BHC (benzene hexachloride, hexachlorocyclohexane) (104), DDT (177) and other materials, (59, 136). These observations are useful enough in providing bald toxicity data but do little to advance the understanding of the way in which these effects are produced.

Paired feeding can be used to determine whether the material is reducing food consumption. The technique consists essentially of feeding the control animal the amount of food eaten by the experimental animal the day before. The method is more tedious and exacting than this summary may suggest. It has been used in a number of toxicity experiments (29, 133, 151, 177, 247) though not always rationally. Thus a paired feeding experiment was carried out to show

that high levels of methyl cellulose (up to 50 per cent of the diet) depressed growth by reducing the amount of actual food consumed simply providing a great bulk of material for the animal to ingest and excrete. A more elaborate experiment is done to explain away a positive finding. This practice stems from the use of factors such as the 100-fold safety margin whereby material intended for use at say 0.2 per cent in human food must be tested at 20 per cent level in the animals diet. Whatever the ethics of adding inert materials to the human diet may be, a material that must be added at concentrations above 10 per cent to the diet of animals in order to produce any effects can scarcely be called toxic. It would seem to be more rational to study the digestion, absorption and excretion of such a material and to examine its effects on the absorption of various nutrients. To feed a material such as methyl cellulose at a level of 30 per cent to rats for two years seems to be the antithesis of a scientific experiment.

3.5. Duration of Tests

The unique property of the chronic toxicity test is its duration. It is reasonable to ask what has been learned from tests lasting a year or more that could not have been learned in tests lasting only three months. Descriptions of chronic toxicity tests rarely indicate the reasons for choosing protracted tests (59, 109, 152). Others contain vague generalizations of the sort "so that the health problems could be properly evaluated" (193, 242, 281, 282) then omit the evaluation. In some cases it is stated that the chronic toxicity data have been obtained to satisfy some administrative body (78, 108).

Most of the designers of ideal tests recommend a duration of two years without attempting to give any reasons for this. Lehman and his associates however, have faced this issue (181, 183, 315) as is shown by the following quotation.

"Will not all aspects of chronic toxicity be demonstrated within twelve months? The answer is an emphatic NO. Experiments in these laboratories have demonstrated many serious effects in the last quarter or third of a twenty-four-month feeding experiment which would not have been observed if the experiments had been terminated earlier. Examples follow:

(a) Bladder stones and enlargement of testes in rats fed certain of the glycols.
(b) Ear tumors in rats chronically poisoned with ergot.
(c) Vitamin E deficiency in rats fed rancid lard.
(d) Liver tumors in rats in chronic selenium poisoning.
(e) Thyroid malfunction in rats fed "P-4000" sweetening agent.
(f) Enlargement of the spleen and liver tumors in rats fed dulcin, an artificial sweetening agent.
(g) Liver tumors in rats fed thiourea" (181).

To the casual reader it may appear that Lehman has made out a good case for the chronic toxicity test. At least it merits serious consideration. Item (a) comes from a paper by Morris, Nelson and Calvery (203) published in 1942. They say "The results of this investigation illustrate clearly the advantage of the long-time chronic toxicity study. The results obtained by continuing the experiment for two years that would not have been noted if it had been discontinued at the

end of one year were the occurrence of urinary calculi in each of the series of animals receiving ethylene glycol and diethylene glycol." This is a surprising statement since it has been known for at least fifty years that ethylene glycol causes oxaluria and renal damage in animals (47). Further, earlier papers have shown that urinary calculi could be produced in rats after 120–130 days (131) and after the even shorter periods of 8–79 days (166). This refutes the conclusion that an experiment lasting two years was necessary to disclose the production of urinary calculi by ethylene glycol, but does not explain why calculi were so tardy in making their appearance in the experiments of Morris *et al.*

Item (b) refers to the production of neurofibromata of the ears of rats after 23 months of ergot feeding at levels of 2–5 per cent (102, 215) and provides an interesting addition to the list of tumours produced by repeated trauma. The test was apparently carried out on the grounds that there was a shortage of animal data on chronic ergot poisoning. It can scarcely be maintained that there is any shortage of data for man (25, 69, 202). Levels of 1 per cent and above of ergot in the diet of man cause the alarming symptoms of acute ergotism (25, 202). The only argument to be extracted from this experiment is that a chronic toxicity test may fail to provide a warning of a very serious toxic hazard to man, intriguing the reader instead with some relatively trivial abnormality.

Item (c) implies incorrectly that the induction of vitamin E deficiency by feeding rancid fat was discovered as the result of a chronic toxicity test. Fitzhugh, Nelson and Calvery (101) in their paper very properly draw attention to the extensive work on the effect of rancid fats in accelerating the destruction of a variety of food factors such as the vitamins, A, D and E and biotin, as reviewed by Burr and Barnes (48). Fitzhugh *et al.* were concerned with pointing out that if rancid fats are included in the diets used in chronic toxicity tests serious complications may be introduced. They point out that the conditioned vitamin E deficiency may take a long time to develop, but only by a complete inversion of the purpose of their paper can it be made to support Lehman's case for very prolonged toxicity testing.

The other examples quoted by Lehman are also open to criticism. Reference should be made to the extensive literature on the toxicity of selenium for man (206, 264) and for animals (206), and to Section 4.5 where the difficulties of assessing the relevance to man of experimental liver tumours such as those induced by selenium (206), dulcin (4-ethoxy-phenylurea) (105) and thiourea (106) are discussed. The reader may be interested enough to examine for himself the evidence of thyroid malfunction in rats fed "P-4000" (1-n-propoxy-2-amino-4-nitrobenzene) (105).

There is very little useful information to be extracted from chronic toxicity tests after the first three to six months. Aspects of this are discussed in Sections 4.1 to 4.6. What information can be culled from the senile survivors of a two year experiment? The ordinary mortality of rats used in chronic toxicity tests is between 70 and 90 per cent at two years. Thus the groups of the size normally used in experiments (10–20 animals) may be reduced to only 2 or 3 animals. There is a remarkable difference in the reaction of workers to the pathological findings in these aged animals.

Some are prepared to accept tumours of the breast, testis, mesenteric nodes, stomach, liver, etc., (152) and abnormalities of ovaries and uterus, mild fatty changes in the liver and tubular degeneration of the kidney (98) as normal manifestations of aging, whereas others attribute lesions such as calcification of the renal pyramids and ovarian enlargement (102) and adenomatous hyperplasia of the liver and focal necrosis of voluntary muscle (98) to the action of the material under test. They may claim to be able to detect an acceleration of normal degenerative processes in kidney (108) and testis (104). Obviously these decisions are made on the relative incidence of abnormalities in experimental and control groups. However, the latter at this time are reduced to a few individuals and do not provide an adequate control. The experimenter must therefore base his conclusions on his knowledge of the normal senile changes in aged rats of his colony. Much that is confusing and conflicting in chronic toxicity tests arises from these studies of rodent geriatrics and many of the conclusions based on the pathological studies of the survivors reflect in turn the skill, experience and prejudices of the pathologist.

4. RESULT OF TOXICITY TESTS AND THEIR INTERPRETATION

4.1. Introduction

In the following sections some detailed consideration is given to the recorded findings in tests for chronic toxicity. Attention is paid only to the non-specific effects which are the usual findings in the more stereotyped chronic toxicity tests. Special investigations are considered later (Sections 5.1 to 5.4).

4.2. Death

As a criterion of toxicity death has the advantages of unambiguity and finality, but it might be suspected of being one of the least sensitive indices of toxicity. It is necessary to establish that death is attributable to the material that has been given to the animal The techniques used to establish the cause of death may themselves give evidence of toxicity. The way in which death has been used as a criterion of toxicity will now be examined.

In Section 2.2 reference was made to the recommendations that chronic toxicity tests should cover the life span of the rat. According to Farris (95) the rat lives about 3 years. Tainter (290) had the patience to follow an experiment for 1004 days until the last rat died, but most workers accept 2 years as the lifespan of the rat and terminate the experiment at this time. The natural death rate of rats varies somewhat from colony to colony but figures of 15 per cent mortality at 1 year, 50 per cent at 18 months and 70–90 per cent at 2 years are usual. The decimation of animals that occurs in a chronic toxicity test is illustrated by the following quotation. "Actually 65 per cent of the rats died by the time two years of feeding had elapsed. Another 12 per cent were killed because of recognizable infections in order to protect cage mates and 19 per cent were dispatched after one year of doses to provide material for histopathological examination and organ weight data. The remainder, or 3.4 per cent, survived until the study was terminated. . . . The causes of the mortality were divided as follows: lung in-

fections 75 per cent, post-partum complications 14 per cent; peritonitis 5 per cent; indeterminate because of autolysis 4 per cent; intestinal infections and intussusception 1.5 per cent. In no instance however did any of these numbers differ significantly between the treated groups and the controls" (59). There are many similar reports in the literature where the material under test has not been shown to affect the natural death rate (108, 152, 187, 242, 273, 274).

Where the death rate is increased the effect may be complex. As Fitzhugh, Nelson and Bliss (100) have pointed out the frequency distribution of deaths may be U-shaped. A larger number of deaths in the first 2–3 months, a fall, and then a steep rise in the number of deaths is a common experience Except where some epidemic has occurred in the colony, these early deaths are usually attributable to the toxic material, and provide an index of toxicity that can be measured with some precision. But the early deaths are really part of a subacute toxicity test and are not here considered in any great detail. A number of factors may lead to the early deaths In Section 3.4.2 it was pointed out that young rats may receive relatively more toxic material than older rats owing to the mode of administration. Young animals may be more susceptible than older animals; the early deaths may weed out the susceptible animals leaving only the more resistant animals, or tolerance may be developed.

In the present context interest is in the second part of the frequency distribution curve, *i.e.*, the deaths occurring from 3 months to 2 years. The natural mortality is often so great that any shortening of life by the toxic material may be difficult to detect. Some workers have reported acute deaths that have not been followed by any increase in mortality in the later stages of the experiment (99, 107, 127, 199). Other workers have found increased deaths in the second year of the experiment with toxic materials such as selenium (100), BHC (104), synthetic sweetening agents (105), mercuriphenyl salts (108), glycols (203), quinacrine (103) and DDT (98). It is not always easy to see from the protocols the grounds for the conclusions, but at least for selenium, BHC and DDT it seems clear that the life-span of the rat has been reduced. However, much earlier indications of toxicity were given for DDT and BHC by outward signs of poisoning (98, 104) and in the case of selenium by acute deaths (100), and it is not clear how much has been learned of toxicity from delayed deaths that could not have been learned earlier and more economically from subacute tests.

Although death might be expected to prove to be an appreciably less sensitive index of toxicity than other criteria such as growth, organ weights, fertility, pathology etc., in a surprising number of tests this has not been the case (59, 98, 100, 102, 104, 107, 199, 290). This is a damaging conclusion indicating that the chronic toxicity test is often crude and insensitive.

4.3. Growth

The effect of toxic substances on normal growth is one of the most commonly used criteria of toxicity but growth has often been studied casually and uncritically. This may be due to the complexity of the problems of growth, and, as the writers have no special knowledge of these subjects, only the limited aspects of

growth used in toxicity testing will be discussed. For other aspects the reader should consult general reviews (89, 307, 320). Almost all the fundamental work on growth has been done on rats, with only an occasional reference to the growth of hamsters (137), guinea pigs (59, 226, 276) mice (226) and dogs (173). The only units commonly used for measuring growth are body weight and organ weights (see Section 4.6), although Smyth and his colleagues (273) have used a fatness index obtained by dividing the body weight by the length of the rat. The usual practice in assessing growth in terms of body weight is to weigh the animals regularly throughout the experiment and either compare visually the weight curves of the various groups, or the average weights of the groups at chosen intervals. The average time taken for the rats to make some arbitrarily chosen gain in weight has also been used (133). These methods make inefficient use of the mass of figures obtained in a chronic toxicity test.

One method of using growth data more fully is that of Zucker (319). When the logarithm of the weight is plotted against the reciprocal of the age a straight line characterizes the normal growth rate. Two important constants are obtained, k which is a measure of the rate of growth and A, a postulated ultimate weight of the rat. Rats continue to grow throughout their life due to unclosed epiphyses (319), and A is the ultimate weight approached asymptotically. The constants, k and A, from a number of different rat colonies are very similar (129, 319). This method of assessing growth has been used occasionally in toxicity testing (29, 184) and merits a more general trial. For one thing it would enable the nutritional state and growth characteristics of the rat colony in any particular experiment to be assessed by the reader, and the results of a toxicity test could be more critically examined. In using the method there is one practical problem arising from the plotting of age as reciprocals. The first week of the experiment is more important in fixing the slope of the line than the second six months. It is unusual to be able to start an experiment with something like a hundred rats all exactly three weeks old and variation of even a few days in the age of the animals will upset the early figures. If rats are bought from a commercial dealer, ignorance of the precise age of the rats may prevent the use of the Zucker method. There are alternate methods of treatment (89, 320) but these have not been used in toxicity testing.

A paper by Fitzhugh, Nelson and Bliss (100) shows that much better use can be made of even relatively unpromising growth data when more rigorous statistical methods are used. Bliss and his colleagues divided the growing period into the first and the second half-year. The rats were fed selenium-bearing grain which, at certain dose levels, produced a steady reduction in growth rate as measured by the average gain in weight of the group. The rats were divided into a susceptible group that died within 8 weeks of the start of the experiment and a resistant group that survived this period. The susceptible group showed a 20 per cent reduction in growth, whereas the resistant group was nearly normal in its growth. This suggests that here growth rate had no advantage over death as an index of toxicity. It brings out the fact that the animals within a group may respond differently and perhaps a more careful study of growth data would

provide a means of recognising this. In the second six months of the experiment the growth rate of rats fed the selenium-bearing grain was significantly lowered. Analysis of co-variance disclosed that the growth rate in the first six months influenced growth in the second six months. If the growth of rats is reduced by various means, and the restraining factor is then removed, the rat quickly regains its normal weight. This can happen even when rats are three years old (208). One way in which the restraint is removed is suggested in Section 3.3.3. When the toxic material is included in the food the dose per kilogram of body weight is much higher in the first few months of the experiment than later.

Other factors that influence the growth rate include the diet. As discussed in Section 3.4.1 it is assumed that the original diet is adequate in most chronic toxicity tests but the common practice of adding the material under test may affect food intake (see Section 3.4.3) and growth. If the diet is rendered unpalatable, or if it produces severe symptoms, the rats may prefer starvation (26, 136). When the material is added to the water, the rats may greatly restrict their water intake even when fed a dry diet (40). Another effect is that of adding to the diet a large amount of relatively inert material—up to 40 per cent of the diet in some cases. The bulk satisfies the rat's appetite, the food intake is lowered and growth is reduced (29, 78, 109, 136, 150, 152, 241). The influence of this factor is usually assessed by measuring food intake and by paired feeding (Section 3.4.2).

In other cases the effect of the toxic agent is more complex. Sulphonamides decreases growth by inhibiting the bacterial flora of the rat. Apparently food intake is reduced, but the addition to the diet of faeces from normal rats restores appetite and leads to normal growth (133) (see Section 3.4.1).

With dinitrophenol (290) and related compounds (280) which raise the metabolic rate it is not surprising that the growth rate is reduced. On the other hand there is no clear reason why a great variety of substances lead to reduced growth when added to the food, *e.g.*, DDT (177), BHC (104), extracts of *Veratrine viride* (127), synthetic sweetening agents (105) etc., or, on inhalation, *e.g.*, 1–3 butadiene (58) and silanes (226). There are anomalies such as the observation that low levels of lead had a greater inhibitory effect on growth than higher levels (205). Further, the effect of some agents is to increase growth. The inhalation of propylene glycol improved the growth of rats (236), and so will the feeding of antibiotics such as aureomycin (90, 163, 283), streptomycin (192, 220, 283), and surface active agents (91). The interaction of two highly complicated processes, drug action and normal growth, produces effects that are very little understood, and needs a fuller and more careful study than it has yet been accorded.

Growth is the end result of a number of complex interdependent processes. An effect on growth may result rather indirectly from the presence of a toxic agent in food and will not necessarily reflect a specific interference with a metabolic process. The value of growth as a sensitive criterion of toxicity clearly depends upon the use of accurate measurements over the period of rapid growth. There seems little point in examining the growth of old and young rats with equal care and labour.

4.4. Increase of Natural Disease

The detection of the effect of a toxic material on the incidence of natural disease will depend on an ability to assess the extent of natural disease in experimental animals. As mentioned in Section 3.5 there is no unanimity about the extent of natural disease in rats and hence the assessment of effects on it are even less well understood. A few reports mention the absence of increase in natural disease (59, 274, 279), others that there is an increase in the natural degenerative processes (108). Robertson and his colleagues (236) found that the inhalation of some glycols reduced the incidence of bronchiectasis in rats and protected mice against infection by airborne bacteria and influenza virus (235, 237).

There is some information on the effect of inhaling irritants on pulmonary disease. Baetjer (23) could not find any certain evidence in the literature that the chronic inhalation of chemical contaminants at low concentrations, of silica dust (24), aluminum dust (301), or bituminous coal dust or smoke (302) altered the resistance of rats to experimentally induced lobar pneumonia. Gardner and his co-workers studied the effects of inhalation of irritant materials on the progress of the pulmonary lesions in guinea pigs infected with an attenuated strain of tubercle bacillus. These infections healed normally, and the rate of recovery was unaffected by nonsiliceous dusts such as marble, coal, gypsum and iron oxide (120, 121) whereas fine silica dusts led rapidly and invariably to death of the animals from tuberculosis (119). Welding fumes (122) caused chemical pneumonia that was often fatal but did not affect the recovery of the survivors from the tuberculous infection.

These papers give some idea of the way in which lung irritants affect bacterial infections of the lung, but do not disclose any simple relationship between irritant action and disease. Less well controlled experiments, such as chronic toxicity tests, are even less likely to disclose any such relationship.

The incidence of diseases other than those due to infection is likely to be so sporadic in animal colonies as to make measurements of the incidence and severity impossible in the small populations used in chronic toxicity tests.

4.5. Pathological Changes

The prominent part that pathological findings play in chronic toxicity tests is a consequence of the role of pathology in providing those observations originally necessary for an understanding of disease. Pathological findings are subjective and will be influenced by the judgment and experience of the man who looks at the organs and histological preparations. Many reports of chronic toxicity tests are prodigal with the pathological lesions they record. Thus, in experimental DDT poisoning, reviews (75, 125) state that lesions have been reported in the skin, heart, lungs, liver, gall bladder, stomach, thyroid, adrenal, testis, voluntary muscle, bone marrow, brain, spinal cord, peripheral nerves and myoneural junctions. It is inherently improbable that one compound would produce lesions in all these tissues, and an attempt must be made to sort the wheat from the chaff.

The first source of difficulty is that of distinguishing naturally occurring disease

and the pathological consequences of ageing (95, 212, 230, 231) from the effects due to the material that is being administered to the animals. This problem can be illustrated by taking three examples of different methods of reporting the large number of lesions found in senile rats. In the first example the authors attribute, in addition to liver lesions, a considerable variety of lesions to the material under test. "The usual 'spontaneous' pathological lesions in control rats are slight even at the end of the two years. Using this as a base line 'selenized' rats showed slight hyperplasia and haemosiderosis of the splenic pulp, slight hyperplasia of the bone marrow, slight focal myocardial fibrosis, and minor renal changes" (100).

In the second example the authors are prepared to accept a considerable variety of changes as normal in senile rats. "There were no correlations between histological findings and the presence of medication. Chronic lung abscesses, varying degrees of liver atrophy of the senile type with narrow cell cords and wide sinuses, and of degenerative changes in the renal tubules were found in many animals. Visible fat drops were present in the periphery of liver lobules in some and spermatogenesis was minimal in most rats" (290).

The third example shows a pathologist disregarding the normal disease processes and looking only for abnormalities attributable to the material under test. "Careful study of stained sections of heart, lung, liver, spleen, pancreas, intestines, stomach, adrenals, kidneys, testes, brain, spinal cord and femoral nerve from 29 rats selected at random from the three generations indicated the complete absence of any toxic manifestations" (109).

It is obvious that there are major differences in approach to pathological findings. The reader, without access to the histological material cannot entirely reject reports of lesions in various organs although his own experience and his judgment of the quality of the work he is consulting may leave him with many doubts. Thus it is well known that the average human myocardium in old age will show much fibrotic scarring but it seems to be much less generally recognised that similar myocardial fibrosis is found in the rat (310). Varying degrees of myocardial fibrosis have been commonly attributed to toxic agents including sulphonamides (116), selenium (100), quinacrine (103), chloroquine (107), DDT (84), and dinitrophenol (147). It is reasonable to ask whether in some of these reports the natural incidence of myocardial fibrosis in rats has been overlooked. The same reserve may be adopted in evaluating reports of splenic siderosis, ulcerative colitis, renal calcification, testicular atrophy, uterine pigmentation and similar senile changes.

Although most reports of pathological findings pass without challenge, examples where disagreement has been expressed illustrate that difficulties are met in interpreting histological findings. Dallemagne and his colleagues (70) described fatty deposits in the kidney of the dog as lesions characteristic of poisoning with BHC. Silver (259) has questioned this on the grounds that fat is normally present in the renal tubules of the dog and that Dallemagne's illustrations appeared to show no more fat than was normal. The second example of dissent in interpretation is the lesion of the liver described by Nelson and his colleagues (180) as due to DDT at levels as low as 5 ppm. It is generally accepted that high

levels of DDT, 300 ppm. and above, will cause liver lesions, but a number of workers have failed to observe liver lesions below 100 ppm. (54, 75, 130). The fine cytoplasmic distinctions that form the basis of Nelson's claim have been made on rat livers fixed in 10 per cent formalin—a notoriously bad cytoplasmic fixation, as Cameron and Cheng (54) have pointed out. These two examples show that conclusions in pathology depend on relatively abstruse points in histology and on technical problems of fixation and staining methods. In interpreting the reports in the literature the reader is called upon to use a considerable discretion and possess a detailed knowledge of methods.

A query as to the value of histological examinations in chronic toxicity tests could be given a variety of answers. One answer would be to compile a list for each compound comparable to that recorded for DDT above. On the other hand most of the reports might be disregarded as insufficiently substantiated. A list of important findings would be a short one including liver tumours induced by selenium, thiourea, dulcin, and butter yellow (dimethylaminoazobenzene), renal damage after ethylene glycol and sulphonamides (263), liver lesions with at least the higher dosages of DDT and BHC, and adrenal necrosis in the dog after DDD (dichlorodiphenyldichloroethane) (217, 218). A vast amount of labour has been expended on these histological examinations. Thus Carpenter, Weil and Smyth (59) prepared 2500 histological sections in one experiment and Fitzhugh *et al.* over 3400 in another (105). Fitzhugh and Nelson (98) by taking about 20 tissues from 213 animals were faced with preparing over 4000 sections, and there are numbers of similar reports (30, 102, 103, 273, 276). The amount of work involved imposes certain limitations. The staining methods must be limited and the examination of the material can only have been cursory.

It is not surprising to learn that Smyth, Carpenter and Weil (275) intend to drop microscopic studies from their range-finding toxicity tests because the technique is not sufficiently sensitive to justify the cost. Bratton (44) has reached a similar conclusion. The routine sectioning of 20 different tissues from each of a series of animals, could probably be replaced with advantage by a careful naked eye examination. Certainly the significant findings listed in the paragraph above could all have been detected by this means. A selection of material could then be made for histological examination which could be more detailed and complete. The vast amount of histological material collected from some toxicity tests seems to have led in turn to improbable catalogues of disorders such as those for DDT given at the start of this section. It would indeed be surprising if some abnormalities, from whatever cause, were not to be found in a collection of 4000 histological preparations from ageing but otherwise normal rats.

In many papers the final conclusion about the freedom from toxicity of a substance appears to rest upon the report of the pathologist that no unusual lesions were observed in the histological preparations from the experimental animals.

4.6. Organ Weights

In biological assays the weight of an organ such as the thyroid for anterior pituitary extracts, or the uterus for oestrones has proved a useful measure of activity (38). In chronic toxicity tests the weights of lungs, liver, kidneys and,

less frequently, spleen, heart, adrenal and brain have been used as criteria of toxicity. The problem of differential growth is a complex one (89) but has been scarcely considered in chronic toxicity tests.

In a number of experiments organ weights have been within normal limits (1, 59, 240, 242, 274, 281) and in others the data have been difficult to interpret (11, 136). The weight of the lungs of animals inhaling silicates (77) or oil fogs (193) has given a useful measure of injury. Atrophy of the testis as measured by weight loss and confirmed by histological examination (151) is acceptable as a toxic effect, but hypertrophy of an organ is much more difficult to interpret. Hodge (151) found that the stomachs of rats fed a diet containing 30 per cent of inert material weighed nearly twice as much as normal, and few would quarrel with his view that this is a case of functional hypertrophy. However, the increase in size and weight of liver and kidneys in animals given trichloroethylene (2), complex glycols (57), DDT (98, 177), BHC (104), lead acetate (205) and an organic silicate (226) have been frequently regarded as unfavourable findings. In some cases this view has been supported by the report of minor histological changes (Section 4.5).

It is doubtful if increased organ weight alone can be accepted as evidence of disease. From Cameron's recent review (53) of the problem of hypertrophy it is clear that many organs, including the kidney and the liver, rapidly hypertrophy when given more work to do. He quotes from Boycott the factors necessary for hypertrophy—good inherent powers of growth, a healthy state of the tissues and an effective stimulus to growth. The toxic material requiring metabolism or excretion supplies the stimulus of work. The occurrence of hypertrophy is itself evidence of a healthy state of the organ and any associated histological change should be reconsidered from this point of view. Certainly the hypertrophied tissues that have been reported in toxicity tests have not been shown to function inefficiently and until they are, few pathologists are likely to accept an increase in weight as evidence of disease.

4.7. Significance of Negative Findings

A negative finding in toxicity testing is very vulnerable to criticism. It is easy to suggest that some positive finding might be obtained by using a different species, different criteria of toxicity, prolongation of the test and so on. Chronic toxicity tests take so much time that these criticisms are likely to remain unanswered, even where their validity may not be questioned. In an experiment with parathion (26) by using growth rate, death and pathological findings as criteria of poisoning it was concluded that 50 ppm. was toxic, but 20 ppm. was not. Subsequent work by Frawley, Hagan and Fitzhugh (112) has shown that at 5 ppm. there is a significant inhibition of red cell and plasma cholinesterase in rats. Although there is difficulty in assessing the weight to be given to this finding, there is no doubt that by the use of a more sensitive index Fitzhugh and his colleagues have produced unequivocal evidence of effects at much lower dose levels than were detected with cruder methods.

This example shows the difficulty of finding acceptable criteria of poisoning or

abnormality attributable to the toxic agent. If statistical conclusions are to be drawn from negative findings there must be confidence in the ability to detect poisoning, and for the purposes of the following discussion it will be assumed that this confidence exists despite the doubts raised in many places in this review. Most chronic toxicity testing seems to be based on finding a dose level that produces no effects in any number of a group of animals. On the other hand those who conduct biological assays assume that there will always be some reactors although the proportion of these may be too small to be detected experimentally when the dose is greatly reduced. This approach is correct and the significance of negative findings in toxicity tests is considered in relation to the number of animals used in the experimental groups.*

Tests should be designed to show what is the greatest quantity of the substance under examination which is practically certain to produce no abnormality in animals receiving the material. This requires two decisions.

1) It is necessary to decide what is the highest rate of abnormality in the experimental groups that can be neglected. This decision is necessary because where a very small proportion of animals responds to a toxic material (one abnormal animal in a million) no experiment is likely to detect the abnormality. If the rate of abnormality is 1 in 1,000 it is still likely to escape detection, if 1 in 100, it may be discovered and if 1 in 10, detection will be relatively easy. It is desirable to detect as low an incidence of abnormality as possible and it is important to know what this incidence is and what rates of abnormality will escape detection in any particular experiment. But the lower the rate of permitted abnormality (*i.e.*, the highest rate of abnormality that will escape detection) is set, the more animals will be required in each experimental group. In practice the limits of abnormality are usually set at 1 in 20, 1 in 40, or 1 in 100. If the toxic material produces abnormalities less frequently than 1 in 100 animals, it would probably escape detection unless at least 500 animals were used at each dose level. This would overwhelm the investigator and his laboratory.

2) Having decided not to attempt the detection of abnormalities occurring less frequently than 1 in 100, it is necessary to calculate the risks of failing by chance to find the abnormalities in the experimental groups. An experiment cannot be devised that will unfailingly detect an abnormality occurring say, in 1 in 100 animals, but the chances of success can be estimated.[1] If the experimental group contains 100 animals, they may all be normal, but if the experiment is repeated a great number of times there would finally be an average frequency of abnormality of 1 in 100 among all the animals used. But it is necessary to know the chances of drawing a blank in any particular experiment.

It is usual to accept such risks of failure at say 1 chance in 20, 1 in 40 or 1 in 100, and it is necessary to decide at which level of permitted abnormality this risk will be taken. Table 2 gives the numbers of animals that must be used when the two variables discussed above are given definite values. If it could be agreed

* In developing these ideas we wish to acknowledge the help of Dr. J. O. Irwin of the Medical Research Council Statistical Research Unit without wishing to hold him responsible for our conclusions.

TABLE 2

Minimum Number of Animals Necessary to Control the Risk of Failure to Detect Abnormals

True frequency of occurrence of abnormally reacting animals	1 in 20			1 in 40			1 in 100		
Maximum permissible probability of failure to detect any abnormality (level of significance)	0.05	0.01	0.001	0.05	0.01	0.001	0.05	0.01	0.001
Least number of animals necessary	58	90	134	118	182	273	295	455	670

that a dose of a substance is fairly well tolerated when it fails to produce a reaction in 19 out of 20 animals, or in other words, poisons only 1 in 20, then either 58, 90 or 134 animals are needed (Col. 1 in Table 2) in order to be more or less confident that this abnormally reacting animal will be found among those examined.

But the chance that this abnormal reactor will not turn up depends upon the size of the group used. If 58 animals are used then there is 1 chance in 20 that there will not be a single abnormal reactor in the group. If 134 animals are used then there is only a 1 in 1,000 chance that no poisoned animals will turn up among the group examined.

If it seems desirable to be more cautious and to say that a substance will only be considered harmless if it poisons no more than 1 in 100 animals, then much larger groups must be used (Col. 3 in Table 2). To be sure that in 19 out of 20 experiments this poisoned animal will be among those examined it will be necessary to use 300 animals.

It may be instructive to consider again the experiments with rats and parathion (26) and the conclusion based on pathological findings on groups of 14 animals. If the true frequency of abnormality was 1 in 20 at the dose level of 20 ppm. negative findings would have been recorded in about 49 out of every 100 such experiments, so that there was about 1 chance in 2 of missing an abnormality occurring even at the high rate of 1 in 20 animals. Alternatively, it can be asked what is the incidence of abnormality that could have been detected with reasonable confidence (*i.e.*, with no greater chance of being wrong than once in 20 such experiments). On this basis it can be said that the abnormality is unlikely to occur in more than 20 per cent of all animals so treated. Of course, the incidence of abnormality may be much lower than this but there is nothing in the experiment to justify a more optimistic prediction. It is legitimate to wonder if it was worth carrying out a chronic toxicity test for a year and preparing some 2000 histological preparations for such an answer.

This is not a unique example of the use of small numbers of animals. Of 100 consecutive experiments recorded in the literature, 66 give the size of groups of rats that have been used and these have been arranged as a histogram (Fig. 1). In recording these groups males and females have been combined, if the sexes

respond differently the size of most of the groups will be halved. Enough has been said to show the statistical weakness of groups of the size normally used.

Another difficulty arises in the form of unexpected findings in experimental groups. In the rats fed parathion at 20 ppm. a malignant tumour of the mediastinum was found in one of the 14 rats examined histologically. Would it be logical to say that parathion at this dose level is a carcinogen for rats? Such tumours occur spontaneously, if rarely, so that here the tumour may be dismissed as unrelated to treatment. How far can any abnormality, whether it is an occasional tumour, the rat with middle ear disease, or even the rat that has been eaten by its cage mates, be ignored if a search is being made for an incidence of abnormality such as 1 in 20. This line of thought is suggested by the designers of tests who

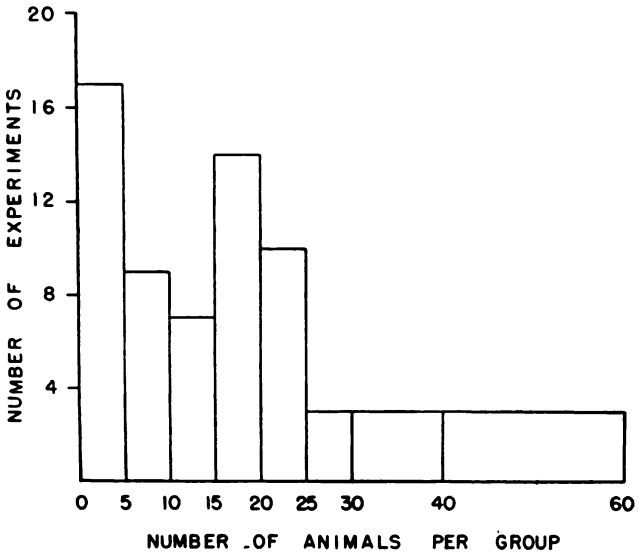

FIG. 1. A summary of information from the published literature showing the numbers of animals used in experimental groups and the frequency with which they were used in experiments on chronic toxicity. Horizontal axis: numbers of animals per group. Vertical axis: number of experiments in which each group size was used.

wish to exclude any possible toxic action of the material under test (see Section 2.2).

There do not seem to have been any experiments carried out on large groups of animals to see whether abnormal reactors do turn up in the numbers expected when low doses of a toxic material have been given. This would only be possible where a sensitive index of response existed (see also Section 6).

5. SPECIAL INVESTIGATIONS

5.1. Studies of Fertility

Deuel and his colleagues (78) have stated that the most sensitive method for the detection of dietary deficiencies and toxicity involves subjecting several

generations of animals to the test diet. The stresses of growth, pregnancy and lactation are all brought to bear on successive generations and certainly have proved to be a severe test of the nutritional value of diets (20, 189, 246, 300). The value of multigeneration studies in chronic toxicity is less certain.

The inclusion of fertility studies in a chronic toxicity test may double or treble the work to be done. Some idea of the extent of the work involved may be obtained by considering a paper by Thienes and his colleagues (295). This study of the chronic effects of nicotine on rats apparently occupied five scientific workers and seven technical assistants from September 1936 until the end of 1942. Their total of 3848 rats does not greatly exceed that of other workers. Deuel *et al.* (78) used 2398 rats, and Carpenter, Weil and Smyth (59) used 1202 litters comprising 9147 young. In studies of this sort it is common to measure the number of litters per female, the size of the litters, the young killed or born dead, the average gain in weight of the growing litters and the number of young weaned. The record-keeping alone is a formidable task requiring special methods.

Quite apart from possibilities such as the disclosing of inadequacies of stock diet and the showing up of genetic abnormalities in the particular strain of rats that is used, it is clear that many practical difficulties are met in fertility studies. Thus Morris, Laug, Morris and Grant (205) found it necessary to try different methods of mating controlled by vaginal smears to determine when the females were in oestrus and whether copulation had occurred. They were able to control their experiments because they used relatively small numbers of animals, 72 females in all, and there seems to be a good case for keeping the number of animals down to manageable proportions. However any experiment of this sort is time consuming and exacting and requires a considerable knowledge of rat breeding (138).

Of the fertility tests that have been done, a large proportion has not shown any effect attributable to the material being investigated (29, 58, 59, 78, 109, 184, 207, 274). Interference with fertility has been recorded for uranium nitrate (199), arsenic trioxide (205), nicotine (295), DDT (97) and ethyl alcohol (284). This effect has been produced at a high dose level where other toxic effects were quite evident. Thus Thienes *et al.* were giving nicotine in twice daily doses at a level just below the acute convulsive dose. The observations that exposure of rats to tetrachloroethylene (56) and carbon tetrachloride (269) caused a slight increase in fertility adds variety to the general picture of reproductive studies.

No doubt negative findings are encouraging evidence of non-toxicity but it is still to be shown that the fertility test is a sensitive index of chronic toxicity. That fertility studies may be useful was suggested by work with parathion (o,o-diethyl-p-nitrophenylthiophosphate) (26). Whereas at dose levels of 20 ppm. and 10 ppm. there was no effect on the life span, food intake, growth or histological findings, fertility was affected. Rats on 20 ppm. had small litters and raised fewer young. At 10 ppm. reproduction was normal in the first generation but the second generation also raised fewer young. The difficulty of assessing the significance of such a finding in a chronic toxicity test was pointed out. In the past two years as the result of the work of Frawley, Hagan and Fitzhugh (112)

with parathion, and personal observations with some other organo-phosphorus compounds, it has become apparent that levels of 20 and 10 ppm. of parathion and other anticholinesterases will produce considerable lowering of blood and tissue cholinesterases. The fertility study therefore may have given the right answer about the chronic toxicity of parathion.

Factors controlling fertility, like those affecting growth, must be numerous and complex. The occasional recording of an unexpected variation (26, 97) may suggest that fertility is a sensitive index that might be of value in this type of test. In our present state of knowledge it is difficult to interpret such findings if they appear to be in conflict with other observations.

5.2. Metabolism of Toxic Substance

Much work has been done on the metabolism of foreign chemical substances introduced into the animal body. The reader is referred to the book by Williams (311), and more than 40 papers by him and his co-workers in the Biochemical Journal. Thorpe and his colleagues have published work on similar lines and have recently discussed the kinetics of these "detoxification" mechanisms (45). Comparatively little work of this kind has been done by those conducting long term studies on toxicity.

In mentioning a few examples, the purpose is to suggest that work along these lines might well lead to the acquisition of a more satisfactory understanding of "toxicity" than will ever be learned from conventional studies of histological or haematological changes in an ageing population of experimental animals.

Yant and his colleagues (318) showed that the ratio of organic to inorganic sulphates in the urine changed very radically when dogs were exposed to benzene. This change was observed long before the blood picture was disturbed. The increase in the amount of organic sulphates represented the excretion of some of the benzene in a "detoxified" form. They found that this "detoxification" was disturbed if the liver of the animals was injured by exposure to carbon tetratetrachloride. The dogs exposed to benzene continued to excrete increased quantities of organic sulphates as long as they were exposed, *e.g.*, up to 250 days.

Studying the excretion of organic sulphates by rabbits exposed to monomethylaniline, Treon *et al.* (298) found that it rose as soon as the animals were exposed but fell again a few weeks later despite continued daily exposure. If the rabbits were removed from monomethylaniline and later re-exposed the same thing happened. No evidence was presented to show that injury to the liver was responsible for this change in the exposed rabbits.

It is natural that dietary factors have been linked with the ability of an animal to metabolize a foreign compound. Rats fed 0.3 per cent biphenyl in a diet containing only 5 per cent protein do not grow as well as controls on a similar low protein diet. If methionine or cysteine were added to the diet containing biphenyl normal growth was resumed (308). The metabolites of biphenyl were not identified but it is not unreasonable to assume that the added amino acids played some part in the mechanism for diposing of biphenyl. Rats fed a high-fat diet are more susceptible to chronic poisoning by trinitrotoluene (TNT) than rats on a high

protein or high carbohydrate diet. On the fat diet, liver lesions were consistently seen (148). It was suggested that the high fat diet prevented the animal from metabolizing TNT adequately because these animals excreted a bright red urine in contrast to the pink urine of rats on the other diets also receiving TNT. The nature of the metabolite was not identified.

In the study of the chronic toxic effects of DDT and other stable chlorinated hydrocarbon insecticides, considerable attention has been paid to the differing tendencies of the compounds to accumulate in the fat both of animals receiving them in their diet and in man (72, 104, 111, 178, 179, 180, 182, 223).

There is no evidence that DDT exerts any toxic effect while it remains in the fat, though its presence in high concentration in the lipoid of the ovary (293) is of interest. Apart from the well-known propensity of heavy metals to be deposited in bone, little has been done on the storage of other toxic compounds in the tissues of animals, and it is difficult to know whether the insecticides are really in a class apart in this particular respect.

Metabolic and chemical studies along these lines obviously offer possibilities of obtaining quantitative data for making comparisons of the toxicities of different materials.

5.3. Carcinogenesis

A major fear of the toxicologist is that some compound that has proved to be relatively non-toxic may subsequently be found to be carcinogenic in man. There is no relationship between the toxicity and carcinogenicity of chemical compounds, and as a rule the toxic dose of a carcinogen is higher than the carcinogenic dose (142). The long delay between exposure and the development of tumours in man is illustrated by the fact that bladder cancer follows 10 to 12 years after exposure to beta-naphthylamine (126), and the bone tumours in radium dial painters took 10 years or more to develop (196). This means that exposure to an unsuspected carcinogen may be extensive and prolonged before the existence of the danger becomes evident. The experimental study of cancer is a highly specialized field. The problem has been largely evaded by the designers of ideal toxicity tests. However, it may be of some help to mention some of the problems as seen by workers outside the field of carcinogenesis, and refer the reader to available reviews (60, 64, 172, 314).

In Sections 3.2, 3.3 and 3.4, above, in the discussion of factors that influence toxicity, mention was made of some effects of sex, age and diet on the development of tumours and these factors will not be discussed further. The route of administration and the solvents and vehicles used (relatively unimportant in chronic toxicity testing) may be of paramount importance in studies of carcinogenesis. It should be remembered that there is a difference in purpose between the cancer investigator on the one hand who is anxious to obtain standardized and reproducible experimental methods to study the mode of action of carcinogens, and the toxicologist who wishes to know if his compounds are carcinogenic under any of the likely conditions of exposure. The methods of administration and the solvents used in studies on chemical carcinogenesis may involve experimental

elaboration beyond the scope of routine toxicity testing. These remarks apply particularly to investigations of the polycylic aromatic hydrocarbons which form the most numerous and most widely studied group of chemical carcinogens. With the polycyclic hydrocarbons tumours are usually obtained after local implantation in the brain, stomach wall, bladder, etc., and in these experiments it has been found that the solvent or vehicle used for administration can enhance or inhibit tumour production (68, 79, 314). The concept of cocarcinogenesis developed by Berenblum (32, 33, 34) postulates two factors acting together, the carcinogen, which converts normal cells into latent tumour cells, and the co-carcinogen, or non-specific irritant, which stimulates the development of the latent tumours. This work suggests that some irritant compounds identified as carcinogens may more strictly be defined as cocarcinogens and the carcinogen may be undetected in the background. Even if attention is limited to carcinogens which can be absorbed from the intestinal canal, *e.g.*, acetylaminofluorene, some other amines and the azo compounds (65), the theoretical and practical difficulties raised by dietary factors may complicate the picture (Section 3.4).

Species differences in susceptibility to chemical carcinogens are very great. The mouse is the animal most widely used, as much for its short life span and high reproductive rate as for its susceptibility to tumours. The rat is less susceptible to some polycyclic hydrocarbons than the mouse but is more susceptible to the azo dyes, whereas the rabbit is refractory to liver tumour formation by the azo dyes. The dog is completely resistant to hydrocarbon carcinogens, but, apart from man, is the only species developing bladder cancer with beta-naphthylamine. Monkeys have been little used for testing chemical carcinogens; at least one attempt to produce tumours in this species was unsuccessful (225). It is not known whether man is more or less susceptible to chemical carcinogens than other species (96, 139). Hartwell (139) has collected data on 1329 compounds that have been tested as possible carcinogens, and 322 of these have been reported as causing malignant tumours. Despite this profusion of carcinogens there is a statement of Salter (249) that only three unadulterated agents have proved carcinogenic to man: radiation, beta-naphthylamine and arsenic. Chemical compounds outstandingly carcinogenic in rodents are usually ineffective in other species, and information on them in man is lacking because the drastic experiments have not been done except in an isolated case (67). At present the only valid evidence of the danger of carcinogenesis in man is carefully analysed epidemiological date (144, 249).

In experimental work on carcinogenesis it has been found that reproducible results bearing a quantitative relationship to the dose can be obtained only with highly inbred strains of mice that are genetically homogenous. These pure strains of mice have the further advantage that the incidence of spontaneous tumours in them is known accurately. As a result a number of strains of mice of varying susceptibility to carcinogens and strains with high and low incidences of particular tumours have become available (17). Such strains of mice have rarely been used in chronic toxicity tests. Smyth and his colleagues reported in 1952 (305) a search for a suspected carcinogen in a chemical industry by epidemiologi-

cal and chemical studies and also by testing the compounds on six strains of mice with a high and low natural incidence of mammary and pulmonary tumours. Lushbaugh *et al.* (193), studying the effect of prolonged exposure to oil fogs, measured the production of lung tumours in a susceptible strain of mice. A paper by Smith, Sunderland and Suguira (267) gives an account of a detailed study of a carcinogenic oil, and the problems of such experiments are discussed. They used ordinary commercial mice successfully, and the statistical methods for determining relative potencies are given in an accompanying paper (36). The papers just quoted exemplify the technical difficulties and the amount of work involved in such a study, and are exceptional in their approach. The usual way in which carcinogens are discovered during chronic toxicity tests is illustrated by the finding of liver tumours in rats fed Dulcin, an artificial sweetening agent (105), in rats fed the alkaloids of *Senecio jacobaea* (66) and the development of neurinomas of the ears of rats fed ergot at high dosage for two years (102). Enough has been said to show the difficulty of applying these findings to man. If the property of carcinogenicity is sought deliberately it may be found more frequently than when the toxicity test is of a general nature. It is extremely difficult to interpret the meaning of the occasional tumour arising in the course of the chronic toxicity test. Most writers are prepared to accept as normal a low incidence of tumours in their experimental animals, especially if some tumours also occur in the control groups. An examination of the statistical considerations in Section 4.7 will show that even a single tumor in one group of 20 animals may be consistent with a relatively high incidence of tumours. Whether it is taken as an indication of carcinogenicity or whether it is rejected as a spontaneous tumour will depend on the judgment of the experimenter. It would be helpful if all such spontaneous tumours arising in the course of chronic toxicity testing were recorded so that those interested in experimental cancer production may investigate the problem further if they feel this to be necessary.

The average chronic toxicity test is an uncertain method of testing a material for carcinogenicity. Negative findings are of little value, and do not mean the compound is not carcinogenic even in well designed carcinogenicity tests (139, 256). If a compound is a suspected carcinogen, tests can probably be done best in an experimental cancer institute, where workers of the necessary experience and training, and suitable materials and animals, are available.

5.4. Skin Sensitisation

Lesions of the skin produced by chemicals are an important cause of loss of working time in industry. The capacity to produce such lesions, even in a small proportion of users, might seriously affect the use of a substance in common consumer goods.

A study of the acute toxicity of a substance frequently includes an examination of its irritant properties and its power to penetrate the skin. It is unusual for the dermal route of absorption to be used to study chronic systemic effects. There are obvious technical difficulties about applying a material repeatedly to the skin of animals who are normally covered with a thick fur.

The main human problem is sensitization. After this has taken place due to earlier exposure, often without any obvious reaction in the skin, exposure to even small quantities of the offending material causes a disproportionately extensive and prolonged response. This is not a manifestation of chronic toxicity in the context of this review.

Techniques for producing sensitization in experimental animals have been described (176), but this type of experiment can be done more readily and inexpensively on large numbers of human subjects, and this will provide a direct answer to the question (271).

6. ASSESSMENT OF HUMAN HAZARDS

The discussion and conclusions are the most unsatisfactory and disappointing sections of many of the papers describing tests for chronic toxicity. It is therefore appropriate that some of the difficulties of interpretation should be outlined. These difficulties may be discussed under the following heads.

(a) Interpretation of positive findings. Much that has been written above points to the difficulties in recognising positive effects attributable to the material under test. False positives may be difficult to avoid. Disturbances of the complex processes growth and fertility involve so many factors that the primary action of the toxic material may not be recognised. Even where the positive effect is simple and unequivocal its significance may be doubtful. In Section 5.2 the storage of DDT in tissue fat was mentioned. Its presence in the tissues registers the fact that DDT has been ingested and absorbed but there is no evidence that its presence in tissue fat exerts any local or generalised toxic effect. The reduction of the blood cholinesterase activity of a rat by 20 per cent is a clear-cut result that may follow the feeding of organophosphorus insecticides but there is not yet any evidence that a lowering of activity to such a degree is in any way injurious.

A positive effect of the first type may reflect a non-specific effect of the material under test, which has only become evident under the particular conditions of the experiment. Comparable observations on man could not therefore be readily made. Specific effects of the second type may be looked for in man but, if found, the interpretation of their significance is no less difficult.

(b) Extrapolation to a safe dose. An ideal toxicity test would demonstrate a positive effect at one dose and an absence of this effect at some lower dose. But in Section 4.7 it was shown that in most experiments a negative finding would mean that the toxic effect was unlikely to occur in more than about 1 in 10 animals. This is scarcely an acceptable risk for man, and every reader may have his own views about what would comprise an acceptable risk. Vaccination against smallpox is associated with a mortality of about 1 in 100,000 which is acceptable for a procedure conferring an obvious benefit upon the individual. It is difficult to extrapolate the findings on the experimental animals in chronic toxicity tests for the risk in any particular case to be estimated.

In dose-response curves there are rarely any experimental data below the response level of 1 in 20, and a study of the frequency distribution below 1 in 20

would require the use of thousands of animals. Hemmingsen (143) used 19,000 mice in insulin assays but stated that very high and very low percentages of response were unreliable and were omitted. That simple extrapolation may be misleading is exemplified by the work of Bliss (37). Studying the effects of carbon disulphide on *Tribolium confusum* he found a straight line relationship between the log dose and response down to the level of 33 per cent response, but below this there was a significant change in the slope. Bliss stated that there was independent evidence that the effect of low doses was qualitatively different from that of high doses. This may be true of the action of toxic substances on higher animals. A practical solution to this problem has been the use of the 100-fold margin of safety by which is meant that substances to be used in food should show no effect in animals when fed at a dose at least 100 times greater than the likely human daily dose. This is a sensible suggestion but its acceptance should not obscure the fact that it has no experimental or theoretical basis. Its introduction has led to some rather irrational experimental practices (Section 3.3.3) and it should not be accepted uncritically in the design of toxicity tests.

(c) Extrapolation from animals to man. An assumption in toxicity testing is that the response of man to toxic agents is sufficiently similar to that of animals to make the experiments worth doing, and the findings derived from them in some way applicable to man. Some dangers in making this assumption have been suggested in discussing species differences in response (Section 3.2.2).

There are remarkably few quantitative data on the effects of toxic agents on man. While it would be possible to quote examples where man was either more or less sensitive than one or more species of animals to the acute effects of different poisons, no generalizations are possible. While man appears to be more sensitive to the chronic effects of fluoride, ergot and beryllium, very little is known about other materials.

Man may appear more sensitive than animals to the effects of a poison because different criteria of toxicity are used. Thus giddiness, headaches and irritability, which are the signs of trichloroethylene poisoning (288) in man, would not be detected in an experimental animal. Many dangerous chemicals are used in industry, and to protect the worker exposed to them maximum allowable concentrations of dusts, mists and vapours in the air have often been specified. These may have been established on the basis of observations made in factories, and especially by studying the conditions under which accidents have occurred (158). Sometimes they have been based on the findings from laboratory tests. Greater weight is given to data however scanty on man (253), and some see very little value in animal experiments designed for this purpose (294). In one instance a suggested permissible concentration was barely safe for rats (86). No allowance was made for the possibility that man might be more sensitive.

If the narcotic doses of a number of solvents (185) for man and animals are compared, the impression is certainly one of uniformity rather than diversity in species sensitivity, but this may not necessarily apply to the remote toxic effects of these chemicals. There is a tendency for the maximum allowable concentration to be lowered progressively as evidence of human poisoning accumulates (132).

This reflects greater experience as a bigger and bigger population is examined, and the detecting of susceptible individuals at the tail end of the frequency distribution curve becomes possible. An idiosyncrasy such as that leading to agranulocytosis would be a serious problem if it occurred with a frequency of 1 in 20. There is no chronic toxicity test that will reveal whether a drug possesses this propensity to produce agranulocytosis. Each compound newly incriminated as its use becomes widespread provides another unpleasant surprise. The problem facing the administrator who controls the addition of chemicals to food is more difficult than that which faces the man responsible for the health of a small working population. The chances of detecting toxic effects or of attributing them to the proper cause becomes extremely difficult in the general population. On the one hand the administrative agency may face charges that many of the ills of the community are due to the presence of chemicals permitted in food. While on the other hand, the addition of more compounds is urged in order that the food industry may continue to meet more effectively the challenge of an increasingly complex civilization. Toxicity tests on animals will not of themselves provide an answer to these problems.

(d) Negative findings. Negative findings are more difficult to interpret than positive findings. Any negative finding is extremely vulnerable to the criticism that a positive answer would have been obtained had the test been done differently. In Section 4.7 the statistical adequacy of negative findings has been discussed. In many cases it would appear that tests with apparently inert materials have been initiated solely on the grounds of administrative expediency as a final trial on some material proposed for use in human food.

Enough has been said to show that there are many difficulties in the interpretation of chronic toxicity experiments and that extrapolation from these experiments on animals to the human population is a matter of guesswork. Unless this is fully recognised the simple chronic toxicity test will be given a status beyond its merits, and the number of tests multiplied to the detriment of more rational methods of studying the possible noxious effects of materials to which man is likely to be exposed.

7. CONCLUSIONS

In their final report to the U. S. Congress, the Delaney Committee investigating the "Use of Chemicals in Food and Cosmetics" expressed their conviction that "chemicals have been utilised in and on the food supply of the Nation without adequate and sufficient testing of their long-range injurious effects" (76). They recommended legislation to ensure that the public was safeguarded by making it compulsory to have adequate tests carried out before an article reached the market. A possible sequel to such legislation would be the adoption of some standard form of toxicity testing. Such a danger was recognised by at least one expert who gave evidence before this Committee when he stated (p. 749) "Investigations of this type make heavy demands upon the general technical knowledge and experience of the investigator whose interest in a problem and whose ingenuity in approaching it should not be thwarted by the neces-

sity of adhering to a stereotyped procedure for the sake of obtaining uniform data on toxicity for comparative purposes," and (p. 757) ". . . I would not wish to be bound by anybody's specifications as to what is necessary to establish the facts with reference to the safety of this or that" (164). This is valid criticism of any stereotyped toxicity test. A second criticism is that the conventional type of chronic toxicity test is not the best way of providing safeguards against human poisoning. The assessment of a toxic hazard can be properly based only on some knowledge of the fate and behaviour of a compound after its introduction into the body. A study of the absorption, distribution and elimination of a compound might take longer and prove more exacting than a routine feeding test. But such work would lead logically to biochemical and physiological studies. This approach would be scientific in contrast to the empirical method of chronic toxicity tests.

The criteria used in the routine type of feeding or inhalation tests are complex and little understood. A disturbance of growth or fertility or even an increased mortality adds little to our knowledge of the mode of action of a toxic material. The value of such criteria is further depreciated by the fact that they are apparently so insensitive as indices of poisoning.

With some knowledge about the behaviour of a compound in laboratory animals, it becomes possible to consider means of finding out how the same compound behaves when given to man. Only when some information of this kind is available would it be worth considering that the results of long-term feeding experiments in animals have any bearing on the problem with regard to man. There would appear to be a much bigger place for the subacute type of test on animals in order to find out just what biological processes are disturbed. Tests of longer duration have little value until something is known about the mode of action of a compound, so that the effects of such an action can, if desired, be studied specifically over long periods. Into this category would fall the tests carried out for carcinogenicity.

The value of routine long-term feeding tests as measures of administrative expediency is not a question for discussion here. The use of such an experimental approach should not be confused with a scientific attack on a difficult problem.

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